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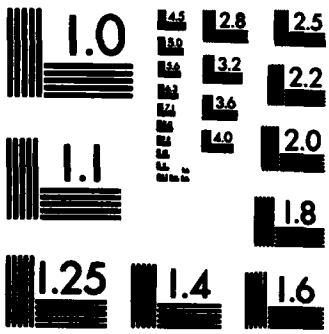

MICROCOPY RESOLUTION TEST CHART
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Research reported here is concerned with an investigation of two new fluorescent techniques for the rapid identification of microorganisms: (1) membrane potential dyes for the discrimination of viable microorganisms in particulate samples, and (2) a proprietary immunofluorescent technique which will allow the individual presence of several different antigens to be detected in a sin e measurement.

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AUGUST 1983

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RAPID IDENTIFICATION OF MICRO-ORGANISMS

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SUMMARY OF RESEARCH

1. OVERVIEW

As outlined in the proposal, we are investigating two new fluorescent techniques for the rapid identification of microorganisms:

(1) We are evaluating membrane potential dyes for the discrimination of viable microorganisms in particulate samples. The fluorescence of membrane potential dyes is dependent upon microbic metabolic activity. Therefore in this technique, if there is a change in the fluorescent signal after the addition of killing reagents, then one can conclude that viable microorganisms are present in the sample.

(2) We are also evaluating a proprietary immunofluorescent technique which will allow the individual presence of several different antigens to be detected in a single measurement. Previous attempts to accomplish this objective have involved the use of different antibodies which were tagged with different fluorescent dyes. As a consequence, the number of different antigen tests which could be multiplexed was just equal to the number of different dyes which could be spectrally separated. This number was generally two or three. Our technique also involves the use of two or more fluorescent dyes, but different antibodies are labelled by using different ratios of these dyes, rather than using a different dye for each type of antibody. In this manner we expect to be able to multiplex as many as 5 or 10 different immunoassays in a single measurement.

2. INSTRUMENT MODIFICATIONS

In the January 1983 progress report we described the Optra flow cytometer which utilizes the optics of a Zeiss epifluorescence microscope. We built a specialized flow chamber and attached it to the microscope stage where the mercury arc lamp provides the source excitation through a 40 X microscope objective. Scattered and/or fluorescent light from the sample is detected by a photomultiplier inserted in the microscope camera tube. The signal pulses can be viewed on the

oscilloscope and are analyzed for pulse height distribution by an Apple computer. We have now added a second (interchangeable) excitation source, a Helium Neon laser (633 nm). The light beam from the laser traverses the flow chamber's capillary tube horizontally and can be easily aligned by mirrors. Fluorescent signals in the far red are detected through the microscope objective and RG665 long pass filters by a red-sensitive photomultiplier tube (RCA 1039) placed in the microscope camera mount. The new RCA photomultiplier does not require a separate high voltage power source; it runs off of a 12V power supply. This design provides the basis for an efficient and economic multiple beam illumination flow cytometer.

3. FLOW CYTOMETER EXPERIMENTS

3.1 Studies with membrane potential dyes, the proposed technique for bacterial detection.

The membrane potential dye hexamethylindodicarbocyanine iodide (DiIC(5)) was proposed for use in distinguishing bacteria from other non-viable particulates. This dye's excitation and emission wavelengths do not interfere with nucleic acid and antibody fluorochromes. Furthermore, DiIC(5) is optimumly excited at 633 nm, the operating wavelength of Helium Neon lasers (this is an inexpensive type of laser). The system was first tested using the dye and the modified instrument with cultured mammalian cells. In these experiments, drugs which altered the cellular membrane potential produced the expected changes in fluorescent signal. *E. coli* stained with DiIC(5) were also detectable in the flow system. The fluorescent signal from the bacteria was not as good as it could be however, and we are presently pursuing histochemical as well as optical solutions to the problem.

3.2 Studies with ratiometric coding of antibodies using multiple fluorescent tags.

Preliminary studies on the proposed technique of labeling antibodies with different ratios of two color fluorochromes were performed in Dr. Shapiro's laboratory. One of the fluorescent labels used in this study is only just now becoming available commercially: phycoerythrin (PE), a fluorochrome derived from algae

(Oi, et, al., J. Cell Biol., 1982, 93:981). The results of this first ratiometric experiment are described below.

Method:

Sample:

Mononuclear cells from human peripheral blood which included helper T lymphocytes, suppressor T lymphocytes, B lymphocytes, and monocytes.

Staining:

A. Hoechst 33342 for DNA (used to gate immunofluorescence measurements)

B. Fluorescent antibodies

1. Labelling molecules

a. Fluorescein (FITC) excited a 488 nm, emission 520-550 nm.

b. Phycoerythrin (PE) excited a 488 nm, emission 570-700 nm.

2. Antibody specificity

a. anti helper T lymphocyte antibodies, mixed 3:1 (PE/FITC):
(PE) 'Leu 3 (Bectin Dickinson)
(FITC) 'T4 (Ortho)

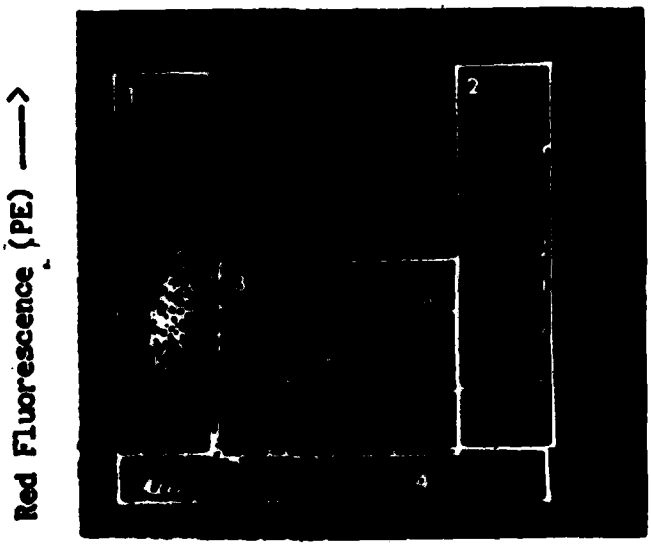
b. anti suppressor T lymphocyte antibodies, mixed 1:3 (PE/FITC):
(PE) 'Leu 2 (Bectin Dickinson)
(FITC) ' T8 (Ortho)

c. anti B lymphocyte antibodies, FITC alone--no PE:
(FITC) ' anti-Ig

Results:

TWO PARAMETER FLUORESCENCE PLOT

1. Helper T Lymphocytes
2. B Lymphocytes
3. Suppressor T Lymphocytes
4. Other cells, including monocytes



Discussion:

The FITC fluorescence on the B lymphocytes, (2) above, was off scale in this plot. These B lymphocytes were easily distinguished from the other cell types because of the higher mean number of fluorescein molecules bound per cell. The two subclasses of T lymphocytes, the helper and suppressor lymphocytes, were well delineated by using the radiometric technique, as the amounts of two fluorescent tags bound per cell defined the cell types. These first results were encouraging. The theoretical analysis of this technique and our program plans will be described in the next section.

4. MULTIPLE TAGGING: DISCUSSION & PLANS

4.1 Introduction

The fundamental notion behind the multiple tagging concept to be explored during this program was the development of a technique for a multiplexed immunofluorescence assay. Normally, immunofluorescent assays are conducted one at a time: the sample is incubated with a fluorescently tagged antisera, and a test is carried out to determine the extent to which the fluorescently tagged antibodies in the antisera have become bound to the antigen whose presence is in question. There are numerous possible tests by means of which this determination can be made, but all of the existing techniques require separate incubations and tests for separate types of antigen. If successful, the proposed multiple tagging technique will allow a number of different antigens to be assayed with a single incubation and test procedure.

The basic objective of the experimental work on multiple tagging will be to determine the number of different antigens which can be tested simultaneously. We plan to carry out the initial experiments using just two different fluorescent tags, and two corresponding spectral detection channels: it is highly desirable to use a single excitation wavelength (or spectral band), and it is difficult to find more than two dyes which can be (a) bound to antibodies without significantly affecting their specificity, and (b) be excited by a common spectral band while emitting in separated fluorescent spectral bands. Our present plan is to use bovine red cells as the antigenic particles, and antibodies (to bovine red cells) which have been tagged with fluorescein and rhodamine. The experiments with these reagents should answer a number of questions, and should allow us to devise further experiments appropriate for more difficult types of antigen.

4.2 Discrimination with a Single Tag

As an introduction to an analysis of multiple tagging, it is useful to consider first the possibilities of single tagging. Let us hypothesize three similar antigenic particles, A, B & C, each of which has N antigenic sites. We further assume the availability of both untagged and fluorescently tagged antibodies to each of these antigens; the mean number of fluorescent

molecules per antibody is M.

Fig.4.1 shows a histogram, or pulse height distribution curve, which might have been obtained by the following procedure: (1) incubate a sample containing antigenic particle A with an excess of tagged antibody to A; (2) separate the bound antigen/antibody complexes from the unbound antibodies (e.g. centrifuge); (3) wash & resuspend the antigen/antibody complexes; and (4) run the solution through a flow cytometer which has been set up to detect the fluorescent tag. The essential features of a histogram of the type shown in fig.1 are: (1) the mean pulse amplitude; (2) the width of the pulse height distribution; and (3) the amount of noise or clutter at low pulse amplitudes. (A measure of the pulse height distribution width is its standard deviation; a more common metric is the Coefficient of Variation, which is the ratio of the mean pulse amplitude to the standard deviation--it is approximately equal to the ratio of the half-width of the distribution, measured at half-max, to the mean of the distribution.)

Referring to fig.1, we see that the mean of the distribution is 8 (arbitrary units, the coefficient of variation (CV) is approximately 20%, and the noise clutter has an exponential cut-off at a pulse height value of approximately 1. (These values are representative of typical measurements with cells.) These parameter values effectively determine the number of different antigen tests which can be multiplexed in a single measurement: in fact, for the example we have selected, this number is approximately 3. To see this, consider the following experimental protocol: the objective is to fill 1/3 of the available antigen sites for antigenic particle A with fluorescently tagged antibodies; 2/3 of the available sites for antigenic particle B; and all of the available sites for antigenic particle C. If this is successfully accomplished, then the identity of an antigenic particle can be measured in a flow cytometer simply by observing the pulse height--as illustrated in fig.4.2. To realize this type of differential tagging, the antibody solution--in which the antigenic particles are incubated--should be made up of the following relative amounts of antibody:

0 parts untagged antibody to A;
1 part untagged antibody to B;
2 parts untagged antibody to C;
1 part tagged antibody C;
2 parts tagged antibody B; and
3 parts tagged antibody A.

The absolute concentration of the antibody solution should be high enough to exceed any anticipated antigen site concentration by a substantial factor (on the order of 10 or more). If equal amounts of antigenic particles A, B and C were incubated in this solution and subsequently assayed with a flow cytometer, the resulting histogram should look like that shown in fig.4.2. We can make the following observations concerning the number of different antigenic particles which can be simultaneously measured in this manner, without excessive ambiguity:

(1) The maximum pulse height is that which is obtained when the antigenic sample is incubated in an excess of tagged antibody, with no untagged antibody present.

(2) The ratio of pulse heights for adjacent pulse height 'bins' is determined by the CV which is measured for a single type of antigenic particle.

(3) The minimum pulse height which can be unambiguously classified is determined by the noise or clutter level.

For a maximum pulse height Pmax, a Noise/Clutter pulse height Pn, and a Coefficient of Variation CV, we can write an approximate expression for the number of unambiguous measurement channels, n:

(4-1) $$n = (1/2)(1 + (\log(Pmax/Pn)/\log(1/(1-1.7CV))))$$.

For the example we have been working with, Pmax = 8; Pn = 1; and CV = 0.2--which gives a value for n of exactly 3.

With reference to fig.4.2 it should be pointed out that equal numbers of antigenic particles A, B and C were assumed (it is the integral of a pulse height distribution curve which is proportional to the number of pulses). It should also be pointed out that if, for a given sample, the pulse amplitude is reduced (e.g. by reducing the power to the light source), then the mean pulse height will be reduced, the width of the pulse

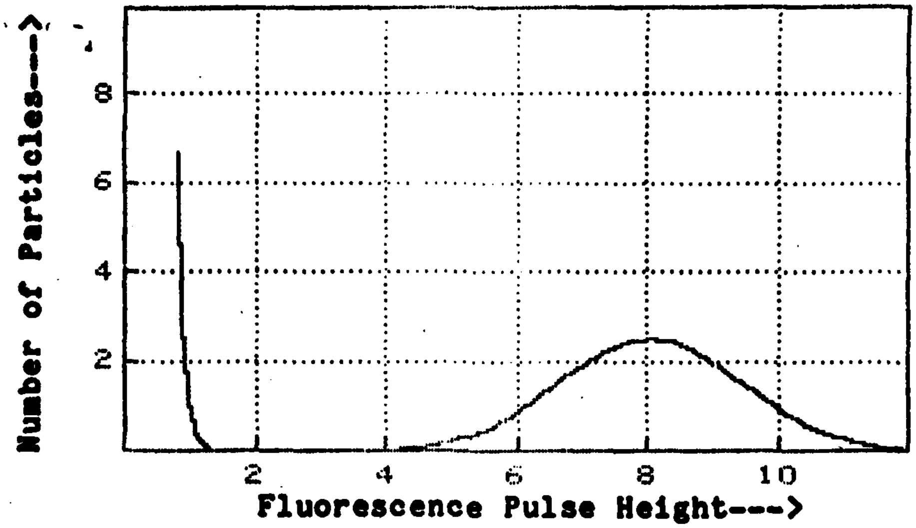


Figure 4.1 Pulse height distribution; Histogram of particles with single tag, single concentration of tag.

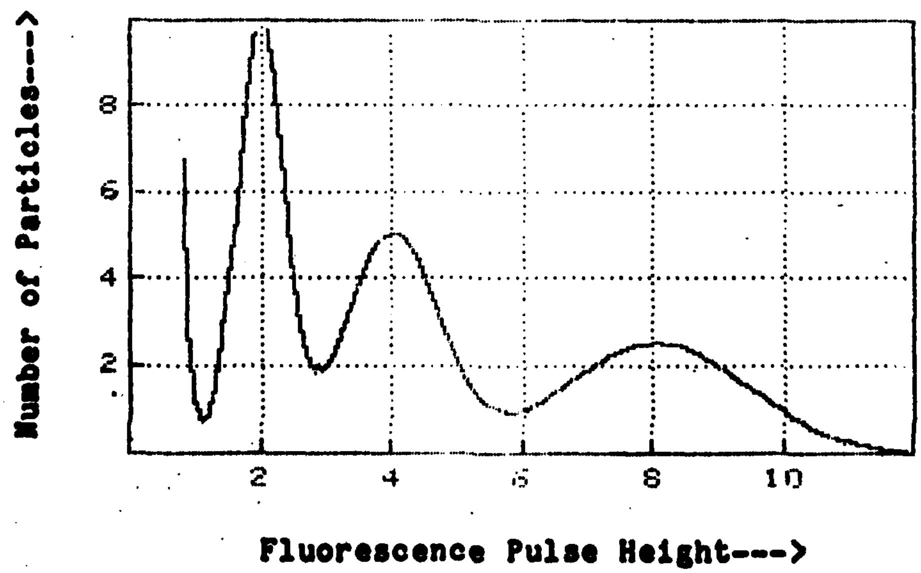


Figure 4.2 Pulse height distribution; Histogram of particles with single tag, three different concentrations of tag.

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height distribution curve will be reduced be the same factor, and the peak height of the distribution will be increased by the same factor.

4.3 Use of 2 Fluorescent Tags

In analyzing the use of two fluorescent tags, it is no longer possible to make use of pulse height histograms of the type illustrated in figures 4.1 and 4.2. Instead it is conventional to use a 2-parameter plot of the type shown in fig.4.3. In such a plot, individual datum points are either plotted individually, or a contour map of data-point density is drawn.

For the sake of simplicity, let us assume that we have N different antigenic particles, each of which has a unique set of antibodies. We also assume that each antigenic particle has the same number of binding sites, that each antibody carries the same number of fluorescent molecules, and that each antigen/antibody complex would be characterized by the same CV. To further simplfy the discussion, and to build on the example provided in section 4.2, we will assume that each of these antigenic particles behaves in same way as A, B or C in section 4.2 (i.e. same noise/clutter level, same peak pulse height, and same CV). We also assume that the fluorescent dyes are essentially similar except for their fluorescence spectra, which are resolvably separated. Given all of these assumptions, we can define the following incubation protocol, which would result in 9 different, resolvable measurements corresponding to the ability to multiplex 9 different immunologic assays:

> Each entry in the following table consists of a letter, which corresponds to 1 of 9 antigen types (A thru I), and three numbers (x/y/z) which correspond to the concentrations of antibodies which have fluorescent tag x (x), fluorescent tag y (y), or no fluorescent tag (z), respectively.

A	0/3/0
B	0/2/1
C	0/1/2
D	1/2/0
E	1/1/1
F	1/0/2
G	2/1/0
H	2/0/1
I	3/0/0

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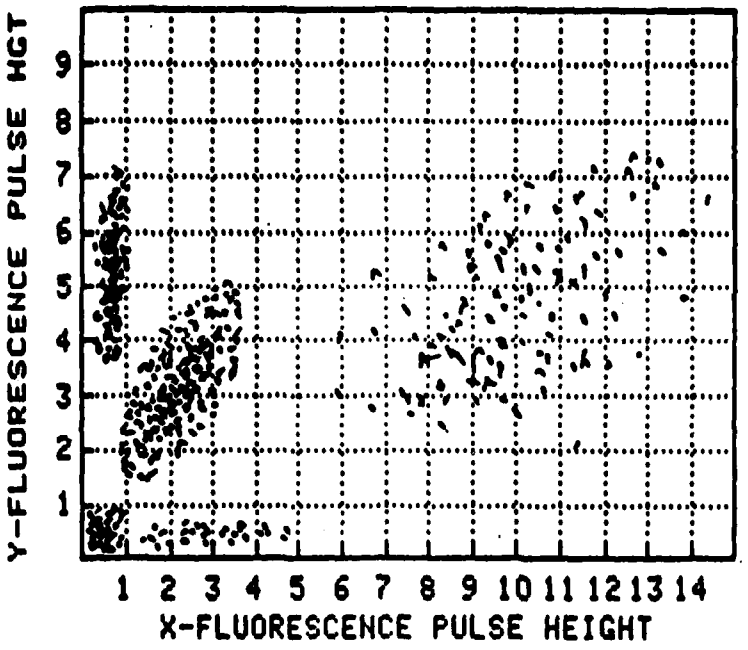

Figure 4.3 2-Parameter pulse distribution plot. Individual data are plotted as points whose coordinates are their fluorescence pulse heights in 2 different spectral channels. (Data can also be plotted as contours depicting data point areal density).

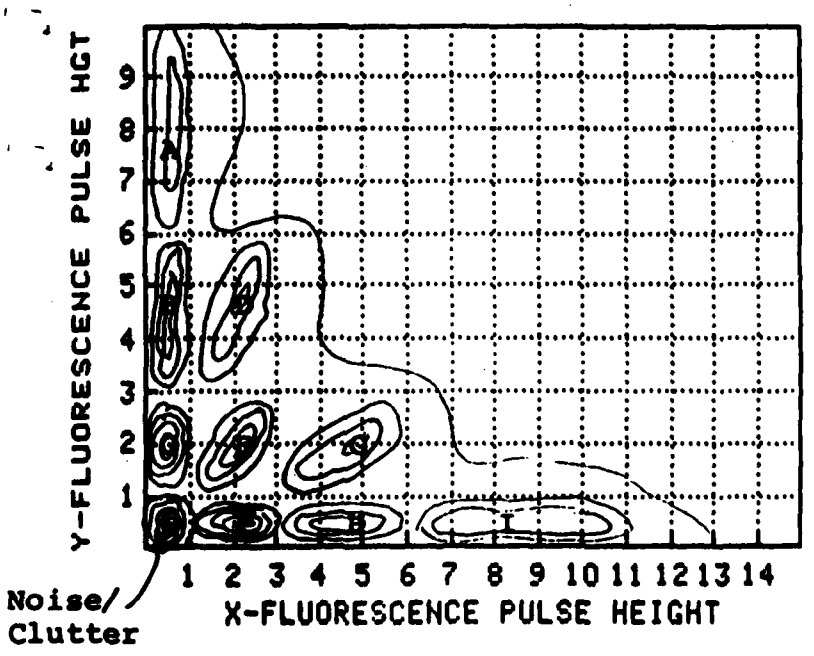

Figure 4.4 2-Parameter pulse distribution plot showing 9 distribution peaks corresponding to different dye tag ratios. Peaks are labelled by antigen type (see text).

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Figure 4.4 is a 2-parameter plot which illustrates the results of a measurement in which equal amounts of all 9 antigenic particles were present. The net result of using 2 different fluorescent tags in this example has been to increase the number of multiplexed measurements from 3 (in the case of a single tag) to 9. In general, if there were n resolution elements in a single-tag assay, then there would be

(4-2) $(n/2)(n+1)-1$

resolution elements in a two-tag assay. By combining equations (4-1) and (4-2) we can write an expression for the number assays which can be multiplexed using two tags, as a function of the peak pulse height, clutter/noise level, and CV which were measured using a single dye tag.

4.4 Planned Experiments

In the multiple tagging experiments planned for the remainder of the year, we will use bovine red cells as the antigenic particles, in combination with commercially available fluorescently tagged (with either fluorescein or rhodamine) and un-tagged antibodies to bovine red cells. The first experiments will serve to establish values for the peak pulse height, clutter/noise level, and CV's which can be obtained with these reagents. We will then separately incubate antigenic samples with the appropriate mixtures of tagged and un-tagged antibodies, separate & wash the incubated complexes, and then mix them before running them on a two channel flow cytometer. The data generated by the flow cytometer will then be processed so that it can be displayed in the form of figure 4.4, and the results compared with theoretical predictions.

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